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Departamento de Biologia

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NDT80 transcription factor as a negative regulator for *Candida parapsilosis* adhesion and biofilm formation

O factor de transcrição NDT80 como um regulador negativo na adesão e formação de biofilmes em *Candida parapsilosis*

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O factor de trancrição NDT80 como regulator negativo da adesão e da formação de biofilme em *Candida parapsilosis*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Isabel Alexandra Marcos Miranda, Investigadora do Serviço e Laboratório de Microbiologia da Faculdade de Medicina da Universidade do Porto e da Professora Doutora Etelvina Maria de Almeida Paula Figueira, Professora e Investigadora do Departamento de Biologia da Universidade de Aveiro.

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Palavras-chave *Candida parapsilosis*, Factores de transcrição, *NDT80*, *UPC2*, adesão e biofilme

Resumo

As infecções por *C. parapsilosis* têm vindo a aumentar nos últimos 20 anos. As suas características intrínsecas de adesão e capacidade de formação de biofilmes são um factor critíco de infecção, sendo os pacientes transplantados ou com o sistema imunitário comprometido ou mesmo os neonatos de baixo peso o grupo de risco mais afectado. Os prestadores de cuidados de saúde são o meio de transmissão mais comum para a infecção por esta levedura. A classe dos antifúngicos azóis são a primeira linha de defesa para tratamento de infecções por este tipo de leveduras. Estes actuam inibindo a enzima lanosterol 14α-demethylase, enzima constituinte da via biossintética do ergosterol. Um estudo recente demonstrontrou que a resistência aos azoles em *C. parapsilosis* poderá ter os mesmos mecanismos observados e estudados em *C. albicans*. Uma estirpe resistente obtida após exposição a Posaconazole revelou uma sobre-expressão de 13 genes envolvidos na biossíntese do ergosterol, entre eles dois factores de transcrição, *Upc2* e *Ndt80*. Com vista a avaliar o papel destes factores de transcrição na resistência aos azoles em *C. parapsilosis*, pretendeu-se efectuar a delecção dos dois genes usando a ferramenta molecular SAT1-flipper cassette. Apenas um alelo do gene *NDT80* foi deletado, originando um fenótipo distinto em comparação com a estirpe original BC014, em particular na sua capacidade de adesão e de formação de biofilmes. Foram realizados testes de susceptibilidade embora sem qualquer diferença evidente entre fenótipos. Estes resultados demonstram que o gene NDT80 pode ser um regulador negativo da capacidade de adesão de *C. parapsilosis*, afectando também o seu potencial de formação de biofilmes.

Keywords

Candida parapsilosis, transcription factors, *NDT80*, *UPC2*, adhesion, biofilm

Abstract

C. parapsilosis infections incidence has been increasing for the past 20 years. Its caracteristics of adhering and forming biofilms are a critical factor for infection caused by this organism, affecting from immunocompromised or transplanted patients to low-birth-weight neonates. The health-care workers are a major transmission vehicle of this fungus. The azoles class of antifungal drugs are the first and most common line of defense to treat infections by this type of yeast species. Its mode of action on the yeast cell works by inhibiting the lanoststerol 14α-demethylase, an enzyme belonging to the ergosterol biosyntethic pathway.. On a recent study it has become clear that *C. parapsilosis* antifungal azole resistance may display similar resistance mechanisms that the ones described for *C. albicans*. A resistant strain obtained after exposure to posaconazole has shown an upregulation of two transcriptional factors, *Upc2* and *Ndt80*. The aim of this work was to assess the role of these two transcriptional factors on *C. parapsilosis* azole resistance. For that, it was intended to knockout both genes using the SAT1-flipper cassette. The strain obtained after disruption of one copy of *NDT80* gene displayed an unexpected phenotype, concerning adhesion and biofilm formation, comparatively to the wild-type BC014 strain. It were also made susceptibility tests although with no evident changes. These results demonstrate that *NDT80* gene may be a negative regulator of *C. parapsilosis* adherence to abiotic and biotic substrates, impairing also biofilm formation.

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IV.Abbreviations List

DNA – *Deoxyribonucleic acid*

Bp – Base pair

BSI – Bloodstream infection

Clo – chloramphenicol

CV- Crystal Violet

DTT – Dithiothreitol

g (mg, µg, ng) – grams (milligram, microgram, nanogram)

g - gravitational force

H_2O – Water

LB – Luria-Bertoli

l (ml,µl) – liter (milliliter, microliter)

mm – millimeters

ms - milliseconds

M (mM, µM) – molar concentration (millimolar, micromolar)

Nou – Nourseothricin

PBS – Phosphate Buffer Saline

PCR- Polymerase Chain Reaction

pm – picomoles

RPMI – Roswell Park Memorial Institute

TF – Transcription factor

U – Units

YPD – yeast peptone dextrose

YPM – yeast peptone maltose

% - percentage

V.Figures and table list

Introduction

Materials and Methods

Results/Discussion

Figure 7. *C. parapsilosis* Cp23 (*NDT80/ndt80*) displayed increased cell to cell adhesion flocculating when growing in YPD liquid media (B) comparatively to the wild-type strain BC014 (A).

Figure 8. *NDT80* aneuploidy increases *C. parapsilosis* adhesion ability. Yeast adhesion ability to polystyrene beads was quantified using Flow Cytometric Microsphere Adhesion Assay. The *NDT80/ndt80* strain displayed approximately five times more adhesion than the wild-type (BC014) strain.

Figure 9. Differences in biofilm formation between *NDT80/ndt80* and the wild-type strain after 24h of incubation at 35ºC on RPMI-1640 medium in a 12-well microplate.

Figure 10. Cp23 strain formed biofilms with increased amount of biomass than BC014. Biofilm formation was measuread using the CV method. Differences in biofilm formation found between Cp23 and wild-type were statistical significant at 24 h, 48 h and 72 h.

Figure 11. Biofilm metabolic activity between Cp23(*NDT80/ndt80) and* BC014. Biofilm metabolic was measuread using the XTT method. There was no significant differences found between *NDT80/ndt80* and the wild-type BC014 strain at 24 h and 48 h.

Figure 12 – *C. parapsilosis* morphological changes triggered by the deletion of one copy of *NDT80* gene. (A) BC014 strain – cells display a predominant round and small morphologic type; (B) *NDT80/ndt80* strain – cells present a bigger and elongated form. Cells were photographed after one day of growth on YPD agar plate at 35ºC.

VI. Introduction

Candida parapsilosis – History and characteristics

C. parapsilosis phylogenetic classification has had undergoing several alterations concerning its pathogenic potential. First discovered by Ashford in 1928, it was isolated from stool of a patient in Costa Rica and named as M*onilla parapsilosis* since it was incapable of fermenting maltose[7]. Described as a non-pathogenic yeast, only in 1940 after being considered the agent responsible for the death of a drug user with endocarditis, it was included in the genus *Candida* and named *C. parapsilosis*[8]. In 2005, this species was subdivided in 3 closely related species, morphologically and genetically; *C. parapsilosis* (*sensu stricto*), *C. orthopsilosis* and *C. metapsilosis*[59], being *C. parapsilosis* (*sensu stricto*) the responsible for the vast majority of clinical infections[9], in comparison with the latter two.

Many of the major human fungal pathogens are known to undergo morphological changes, which in certain cases are associated with virulence. *C. parapsilosis* colonies display diversified shapes; oval, round and cilindric, having in common the white milky colour. They do not form true hyphae and only exist in two morphogenic forms: yeast or pseudohyphae, in contrast with other *Candida* species, like *C. albicans*[36]. In the last twenty years, it is known that infection by this pathogen has increased. So, it is of the most importance to describe the epidemiology, virulence traits, clinical manifestations, genetics, and antimicrobial susceptibility of *C. parapsilosis* to provide a broad and up-to-date reference for this pathogen[10].

Prevalence

Invasive yeast and mould infections in immunocompromised patients have consistently shown a lower incidence in comparison with bacterial infections, in particular on the hospital environment[1].

Infections by these pathogens are increasing and have been associated with a significant increase in the number of individuals receiving immunosuppressive therapies, such as cancer patients on chemotherapy and organ transplant recipients[37,38].

In 2003, a surveillance program was conducted in Europe, in particular on Nordic countries, in order to study the prevalence of *Candida* spp. in the period of time of two years (2004-2006)[2]. The incidence of these species was more than 50% of total hospital infections, being *Candida albicans* the most common cause of infection.

As one of the most virulent *Candida* species, infections by *Candida parapsilosis* have became more prevalent in the last twenty years, being therefore actually considered an emergent fungal pathogen. In some cases it is the most common species on fungal bloodstream infections (BSI) in the nosocomial environment[60], even surpassing *C. albicans*[3,4], frequently associated with disease and even death, in particular in infants and immunocompromised patients. Nevertheless, *C. parapsilosis* can subsist in the human organism being a commensal pathogen, particularly in the human skin, with limited pathogencity by intact integument.

The increment of invasive candidiasis infections is due to instrinsic organism factors. This increasing trend of *C. parapsilosis* has been attributed to several factors including the organism's growth capacity, its affinity for intravascular devices and prosthetic materials, gastrointestinal colonization and transmission from the colonized hands of healthcare workers[10,44,46]. The affinity of *C. parapsilosis* for foreign material is shown by infections related to peritoneal dialysis catheters and prostheticheart valves, and this characteristic may be important in infections of cancer patients with indwelling access devices[52,54], as well on neonatal intensive care units[56,57].

Antifungal therapy

In order to treat cases of candidiasis, several antifungal agents have been used through the past until nowadays. The development of new therapeutic forms of treatment has been enhanced on the last few years, in particular on a molecular level, but they are not yet an effective and somewhat limited alternative to classic antifungal treatment.

There are at least 5 classes of antifungals that are most common on the clinical environment to treat *Candida* spp. infections, including polyenes, echinocandins, nucleoside analogs , and azoles[49].

Within these classes, the azoles are the most commonly used on medical practice due to their broad spectrum and effectiveness. This specific class has been in constant evolution, having the first azole marketed (ketoconazole) on 1958, and since then, numerous other azoles have been available commercially, the latter one on 2005 (posaconazole).

The azole class of antifungals work by inhibiting the lanosterol 14α-demethylase, an enzyme that belongs to cytochrome P450. This enzyme has a key role in the ergosterol synthesis, and is encoded by the *ERG11* gene[18] (**Figure 1**)

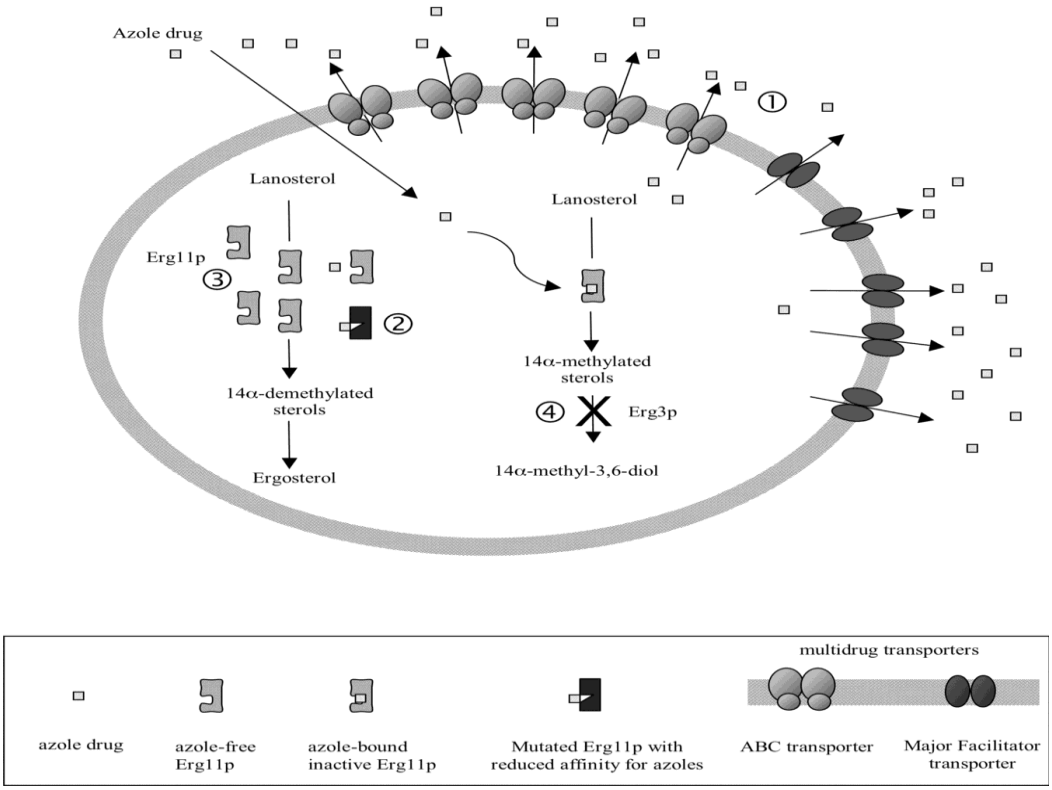

Figure 1 Azole antifungal action mechanism – The azole drug enters the fungal cell and inhibites the 14α-demethylation of lanosterol, leading to the accumulation of methylated sterols on the interior of the cell, ultimately causing cell death. (http://www.biochemsoctrans.org/bst/033/1210/bst0331210f01.htm?resolution=HIGH)

Up to this date, little is known about the molecular mechanisms underlying the *C. parapsilosis* pathogenicity, acting *C. albicans* as a "comparison species" due its extensive knowledge regarding its molecular and genomic caracteristics.

Molecular mechanisms of azole resistance

Yeasts are, like the most part of microorganisms, adaptable to specific types of conditions of stress, whether they are pH, temperature, saline and even oxidation related. It can alter their genome to better withstand these conditions, and in the process develop mechanisms of resistance to antifungal drugs, namely azoles[11]. In recent years, intense research has been conducted, leading to a better understanding of the molecular mechanisms of azole resistance, among which the most prevalent in *C. albicans* are: (i) reducing intracellular

concentration of the azole drugs by overexpression of efflux pumps belonging to either the ATP-binding cassette superfamily (*CDR1*, *CDR2*) or the major facilitator superfamily (*MDR1*); (ii) up-regulation of the *ERG11* gene encoding 14α-lanosterol demethylase, the primary target of azole drugs; and (iii) point mutations in *ERG11* causing amino acid changes that lead to decreased affinity for azole drugs[19]. These mechanisms can combine and even have a synergistic effect on a single isolate[20].

The hemoprotein *ERG11p* is the azole target in *Candida* spp.. It has the function of fixing oxygen atoms, needed to the enzymatic reactions[24]. This protein is involved in the ergosterol biosynthetic pathway of the fungi by demethylating lanosterol and catalyzing the first step in ergosterol synthesis[27]. With azole exposure, the ergosterol pathway is inhibited, and methylated sterols such as 14α-metil-3,6-diol have increased intracellular accumulation, consequently inhibiting the cellular growth.

Alterations on the ergosterol biosynthetic pathway can induce azole resistance. On *C. albicans* the decrease of intracellular drug accumulation may be caused by overexpression of the efflux pump genes, and mutations in *ERG11* gene. They are described as being among the most common mechanisms of azole resistance[28].

The exposure of *C. albicans* to azoles is also known to result in accumulation of 14-methylfecosterol, which is subsequently converted by the sterol 5,6-desaturase to 14-methyl-ergosta-8,24 (28)dien-6-diol (14-methyl-3,6-diol). Since 14-methylfecosterol is capable of supporting fungal growth and 14-methyl-3,6-diol is toxic to the cell, inactivation of the sterol 5,6-desaturase renders the cells resistant to azoles.This is described as a third possible mechanism of resistance in *Candida* spp.[25].

Azoles are a fungistatic drug class, this is an important factor to consider in resistant strains, providing a selective pressure to the organism to become resistant, developing mechanisms in which the gain-of-function mutation rate is significantly higher. Thus a significant and increased number of azole-resistant strains can be yielded[29].

Regulation of transcription factors (TF)

Up to date, the transcriptional factors of the zinc cluster family have been identified as regulators of the transcriptional control of azole resistance in *C. albicans*. They mediate the upregulation of the ergosterol biosynthesis genes, acquiring called "gain-of-function" mutations. Azoles exposure trigger mutations, either in transcription factors or in the azole target gene. Once acquired, transcription factors (TF) become hyperactive, increasing the expression of its regulated-target genes, such as *ERG11* gene and the multidrug efflux pumps of the ABC transporter and other major facilitator superfamilies[13].

In *C. albicans*, *Upc2p* has been described as a TF that targets genes of the ergosterol biosynthesis pathway and it is correlated as an important factor of regulation of the *ERG11* gene. Mcpherson *et al*.[22] identified *UPC2* as an homologue of *ECM22*, an important gene involved in the ergosterol biosynthesis in *Saccharomyces cerevisae*. Furthermore, they have found that the deletion of *UPC2* gene has lead to impaired growth under anaerobic conditions and high susceptibility to azoles. Conversely, an overexpression of *Upc2*p cause an increased resistance to these drugs.

Furthermore, Dunkel *et al.*[31] showed that azole resistance is linked to a gain-of function mutation in the sterol metabolism regulator *Upc2*p, leading to constitutive expression of ergosterol biosynthesis genes.

The *Ndt80p* TF in *C. albicans* controls positively the expression of the gene encoding drug efflux pump *CDR1*[30]. It has been described that *Ndt80p* is required for the hyphal growth in response to different filament-inducing cues and for the proper expression of the filamentous transcriptional genes program which characterize this type of growth, such as the chitinase *Cht3p* and the cell wall glucosidase *Sun41p*[33]. Moreover, *Ndt80p* was found on the promoters of ergosterol biosynthesis genes, including on the azole target *ERG11p*.

Case of study

Despite improvement of antifungal therapies over the last 30 years, antifungal resistance is a major concern on the clinical practice[34].

On Silva *et al*.[35], it becomes clear that *C. parapsilosis* antifungal azole resistance may have the same resistance mechanisms that are underlying *C. albicans* resistance, in particular the activation of efflux pumps by the upregulation of *MDR1* gene.

On this study, an azole-susceptible strain (BC014) was *in vitro* exposed, for a maximum of 60 days, to a constant concentration of three different azoles: fluconazole, voriconazole and posaconazole, separately. A set of resistant strains was generated.

The resistant strain obtained after exposure to posaconazole has shown an upregulation of 13 genes involved in ergosterol biosynthesis, and two transcriptional factors, *UPC2* and *NDT80*. The two upregulated TF are well documented and identified in *C. albicans*, and are correlated with the overexpression of *ERG* genes. The results of this study were the basis for this experimental work, to better understand the roles of these two TF in *C. parapsilosis.*

VII.Objectives

This experimental has the main goal to assess the role of both *NDT80* and *UPC2* genes in azole resistance in *C. parapsilosis*.

VIII.Materials and Methods

Growth conditions

Escherichia coli – DH5α

Competent cells of *E.coli* DH5α were used for plasmid construction and cloning. They were grown in liquid or solid (containing 2% agar) Luria-Bertoli (LB) medium. Positive clones were grown in the same medium supplemented with chloranphenicol (Clo) to a final concentration of 25 µg ml^{-1} and were later stored at -80ºC in LB containing 20% glycerol.

C. parapsilosis - BC014

C. parapsilosis BC014 strain was grown in liquid YPD (*Yeast Peptone Dextrose*) medium (1% yeast extract, 2% peptone, 2% dextrose) or in YPD solid (containing 2% agar). For transformants selection YPD medium was supplemented with Nourseotricin to a final concentration of 200 μg ml^{-1} (Nou 200).

All *C.parapsilosis* transformants were later stored in YPD medium with 40% glycerol, at -80ºC.

E. coli competent cells preparation

E. coli DH5α was incubated at 37ºC overnight and inoculated in 5 ml of liquid LB medium until reaching an optic density (OD_{550nm}) of 0.3. Then, 4 ml of the culture was inoculated in 100 mL of LB medium and *E. coli* was grown under the same conditions until reaching a similar OD_{550nm}. Afterwards, it was put on ice for 5 minutes and centrifuged at 1,700 *g* for 5 minutes at a temperature of 4ºC. The pellet was then ressuspended in 40 ml of ice-cold TFBI solution (30mM KOAc, 50mM $MnCl_2$, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 15% glicerol) and again centrifuged at 665 *g* for 5 minutes at 4ºC. Finally, pellet was ressuspended in 5 ml of ice cold TFBII solution (10mM NaMOPS pH 7.0, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% glicerol), incubated on ice for 5 minutes and gently divided in 200 μl aliquots in Eppendorf tubes and stored at -80ºC until use.

Plasmid Construction

The pCD8 plasmid, gently gifted by Prof. Geraldine Butler, contains the SAT1 gene which confers resistance to nourseotricin (Nou). This plasmid was used to develop the *NDT80* and *UPC2* disruption cassetes as described by *Ding et al.*[14]. Upstream and downstream sequences of both genes were amplified by PCR as described in Table 1, using as template the using *C. parapsilosis* BC014 genomic DNA.

Upstream sequence of the *NDT80* gene was amplified through the following PCR program: 95ºC for 2 minutes for denaturation, followed by 30 cycles of 95ºC, 51ºC, 72ºC for 30 seconds each, with the final extension step of 72ºC for 10 minutes. For the downstream sequence the PCR program comprised a 95ºC denaturation step for 2 minutes , followed by 30 cycles of 95ºC, 63ºC, 72ºC for 30 seconds each, with the final extension step of 72ºC for 10 minutes.

The *UPC2* fragments cloned into the pCD8 plasmid were amplified by PCR with the primers *UPC2*_up_F and *UPC2*_up_R; *UPC2*_down_F and *UPC2*_down_R, .

To amplify the upstream region of the *UPC2* gene (491 bp) , the temperature profile used was: 95ºC for 2 minutes for denaturation, followed by 30 cycles of 95ºC, 65ºC, 72ºC for 30 seconds each, with the final extension step of 72ºC for 10 minutes. For the downstream fragment (463 bp), PCR program comprised a 95ºC denaturation step for 2 minutes, followed by 30 cycles of 95ºC, 57ºC, 72ºC for 30s each, with the final extension step of 72ºC for 10 minutes (**Table 1**).

Table 1: Components and concentrations used in PCR to amplify both upstream and downstream regions of the *NDT80* and *UPC2* genes

Reagents	**Final Concentration**	**Final Volume**
Taq Buffer 10x	1x	2,5µl
dNTP	0,2 mM	0,5µl
Primer_F (up/down)	4 pmol	1µl
Primer_R (up/down)	4 pmol	1µl
H_2O	Adjusted to 25 µl	
Taq DNA Polymerase	1 U	0,2µl
Genomic DNA	About 100 ng	

Both upstream and downstream sequences of the *NDT80* and *UPC2* genes were amplified by PCR using primers designed in the OligoExplorer program and listed on **Table 2.** PCR reactions were carried out in a BioRad MyCycler Personal Thermal Cycler® and its products were confirmed for its correct size by electrophoresis in 0,8% agarose gel.

Table 2. Primers used to amplify the upstream and downstream sequences of the *C. parapsilosis NDT80* and *UPC2* genes

Primer	Sequence (5'-3')
NDT80_up_F	GGG GGT ACC GGC AAT TTT GAT TTT TGG GTT C
NDT80_up_R	GGG GGG CCC GAG GCA CCA CCA GCA GTA GAG T
NDT80_down_F	TCC CCG CGG GAT GGG AGA AAA AAC TGA ACC TTG
NDT80_down_R	CGA GCT CAG ATG GCA TTG TAG TCA GTA GCA TC
*UPC2*_up_F	GGGGGTACCTCTCGTGTCTTGATTTTTTGTGTAG
*UPC2*_up_R	GGGGGGCCCGCTTGTTCTCCTTGCGTGTGTATTA
*UPC2*_down_F	AAAAAGCGGCCGCATGGATGGGAAATGGATACATG
*UPC2*_down_R	gggGAGCTCTTCGTTGATGATAGAAAAGTTAGGA

Finally, fragments were digested with *KpnI* and *ApaI*, in the case of upstream fragments, and *SacII*/*SacI*, in the case of downstream fragments. These fragments were ligated sequentially to the digested plasmid.

Plasmid DNA *E.coli* extraction

Plasmids used were extracted from *E. coli* DH5α according to NZY Miniprep Kit protocol (NZYtech®).

DNA agarose gel electrophoresis

DNA electrophoresis was performed using a constant voltage of 80 V (Power Pac Basic, Bio-Rad®) for 60 min, running in TAE buffer 1x (0.04 M Tris, 50 mM EDTA pH 8, 1 M glacial acetic acid), in a 0,8% agarose gel (Ultra Pure Grade NZYtech®) incorporating ethidium bromide (0,5 µg/mL). DNA samples were loaded on agarose gel mixed with 1x loading buffer (10 mM Tris-HCl (pH 7.6); 0,03% bromophenol blue; 60% glycerol; 60 mM EDTA). DNA was visualized and analyzed in a GelDocXR equipped with Imaging System from BioRad®.

Plasmid DNA restriction

For fragment cloning, approximately 3 µg of plasmid DNA were used with about 3 Units (U) of each restriction enzyme (*KpnI*/*ApaI* and *SacII*/*SacI*). Reactions with Fast Digest enzymes (*KpnI*/*ApaI*) were incubated at 37ºC for a maximum of 20 minutes, whilst reactions with conventional restriction enzymes (*SacII*/*SacI*) were incubated for a period of 16 hours at 37ºC. The linearized plasmid product was then purified using QIAquick Nucleotide Removal Kit (Qiagen) according to the manufacturer protocol.

Plasmid DNA Desphosphorilation

About 1 µg of the digested purified plasmid was desphosphorilated by 1U of alkaline phosphatase (AP) in a reaction of 30 µl containing AP buffer 1x (0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 8.5, at 37ºC for 30 minutes and 2 hours. AP inactivation occurred at 65ºC for 10 minutes.

DNA Ligation

DNA fragment ligation to a restricted desphophorylated plasmid was carried out by T4 DNA Ligase enzyme (Fermentas). Ligation reation included approximately 30-60 pmol of the plasmid, 90-180 pmol of DNA fragment, T4 DNA Ligase buffer 1x (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% polietilene glicol 8000), 1 U of T4 DNA Ligase and H_2O MilliQ. Inactivation of T4 DNA Ligase occurred at 65ºC for 10 minutes.

E.coli Transformation

For DNA cloning, competent *E.coli* DH5α cells were transformed with ligation reaction. For that, 200 µl of DH5α cells were mixed with 5 µl of the ligation reaction and incubated for a period not exceeding 30 minutes. A negative control, without DNA was performed. Posteriorly, a heat shock treatment at 42ºC for 90 seconds was given to the mixture, followed by incubation on ice for 2 minutes. Then it was added 800µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 2.0 mM glucose) and incubated for 1 hour at 35ºC to allow cells to recover. Following a centrifugation for 1 minute at 660 *g* at room temperature, about 50µl of the cell mixture was plated onto LB agar medium supplemented with Clo 25 mg ml^{-1}. Plates were incubated overnight at 37ºC.

The grown colonies of DH5α containing plasmid of interest were inoculated and grown overnight for 37ºC in 5 ml of LB medium supplemented with 25 mg ml^{-1} Clo for plasmid DNA extraction.

E. coli DH5α clone plasmid DNA extraction

From the cell suspension resulting of the described above, about 4 ml of the culture were centrifuged at 18,000 *g* at room temperature for 5 minutes. The pellet was washed with 1 ml of Solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) and vortexed, followed by a centrifugation at room temperature at 18,000 *g* for 5 minutes. The supernatant was removed and the pellet ressuspended in 100 µl of the Solution I, then 200 µl of solution II (0.2 M NaOH, 1% SDS) and 150 µl of ice-cold Solution III (3M Potassium acetate pH 5.0) were added and mixed by inversion. Following an incubation on ice for 5 minutes, the mixture was then centrifuged at 4ºC for 10 minutes and the supernatant transferred to a new microtube. To the supernatant it was added 0.7 vol. of isopropanol and left to incubate for 10 minutes, followed by a centrifugation at 18,000 *g* at 10ºC for 10 minutes. The resulting pellet was then washed with ethanol 70% and again centrifuged at 18,000 *g* for 10 minutes at 4ºC. The aqueous phase was removed and the pellet dried for 30 minutes at 37ºC and ressuspended in sterile H_2O with 10 mg ml^{-1} RNAse A (NZYtech®).

Clone Screening

Plasmid DNA extracted was used as template in a PCR to screen for positive clones. Specific primers were used (**Table 2**) to confirm the presence of upstream and downstream sequences of *UPC2* and *NDT80* genes in the construction of the disruption cassettes. Nucleotide sequence of the cloned fragments were confirmed by sequencing. Thus, two disruption cassette for *UPC2* and *NDT80* genes were obtained, pNG3 and pNG4, respectively.

Amplification of the disruption cassette

Disruption cassettes in plasmids pNG3 and pNG4 were amplified by PCR using the pairs of primers *UPC2*_up_F/*UPC2*_down_R and NDT80_up_F/NDT80_down_R, respectively. PCR reactions were prepared as described in **Table 3** and PCR program was carried out in a BioRad MyCycler Personal Thermal Cycler® as described above. PCR products were visualized after DNA electrophoresis in 0,8% agarose gel and purified using QIAquick PCR purification Kit (QIAGEN). The PCR amplification temperature profile for both plasmids comprised a 95ºC denaturation step for 5 minutes , followed by 30 cycles of 98ºC, followed by an extension step at 65ºC for 2:20 minutes for pNG3 and 2:30 for pNG4, with the final extension step of 72ºC for 5 minutes.

Table 3. PCR reaction mix prepared to amplify disruption cassette in pNG3 and pNG4 plasmids.

Reagents	Stock Volume	Final Concentration
H_2O milliQ	Adjusted to 25 µl	
Primer F (10 µM)	0,75 µl	0.3 µM
Primer R (10 µM)	0,75µl	0.3 µM
DNA	1µl	100 ng μl^{-1}
KAPA HiFi Hot Start *ReadyMix*	0,2 µl	1x

C. parapsilosis Transformation by electroporation

C. parapsilosis transformation protocol was adapted from Zemanova *et al.*[16] with minor adjustments. A BC014 *C. parapsilosis* colony was inoculated overnight in 85 ml YPD medium at 30ºC until reaching an OD_{600} of approximately 1.8-2.0. Cells were collected by centrifugation at 5000 *g* for 5 minutes. Then, the pellet was suspended in 10 ml of Tris-EDTA buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) containing 10 mM of ditiotreitol (DTT). After, yeast suspension incubation at 30ºC for 1 hour, with agitation (100 rpm), it was added 40 mL of ice-cold distilled water, and transferred to a clean cold 50 mL falcon which was centrifuged for 5 minutes at 5,000 *g*. Supernatant was discarded and pellet was washed twice , first with 50 ml of cold destilled water and then with 10 ml of ice-cold 1 M sorbitol. After removing the supernatant, competent cells were ressuspended in 125 µl of 1 M sorbitol, divided in aliquots of 50 µl, mixed with approximately 1 µg of disruption cassette and incubated for 5 minutes on ice. A negative control without DNA was made.

Mixture was transferred into a 1mm electroporation cuvette and transformed at 1,25 kV for 5 ms in a Gene Pulser X-cell (Bio-Rad) electroporator. After electroporation, cells were imediatelly suspended in 950µl of YPD medium containing 1 M Sorbitol and allowed to recover for 4 hours at 30ºC with agitation (180 rpm). Finally, 100 µl of the mixture were washed and plated onto YPD agar medium supplemented with Nou 200 µg ml^{-1}. Transformants were obtained after 48-72 hours of incubation at 30ºC.

SAT1-Flipper cassette genome integration - screening and recycling

In order to confirm disruption cassete integration in the target gene locus, clones grown in Nou were screened by PCR. Primers used were designed to amplify genomic DNA region adjacent to the location of the gene (CpNDT80gen_F or Cp*UPC2*gen_F) (**Table 5**), and the FLP recombinase region located within the SAT1-Flipper cassete (FLP_R). Reactions were prepared as described in Table 2 and the following PCR program: 95ºC for 3 minutes; 30 cycles of 95ºC for 30 seconds, 66ºC for 30 seconds and 72ºC for 3 minutes, ending with an extension step at 72ºC for 10 minutes, was used. PCR reactions were carried out in a BioRad MyCycler Personal Thermal Cycler®. Products reactions were runned in a electrophoresis in 0,8% agarose gel.

Table 5. – Primers used for screening disruption cassette integration at its target genomic locus. CpNDT80gen_F and Cp*UPC2*gen_F comprise the region adjacent to the location of *NDT80* and *UPC2* genes, respectively. FLP_R belongs to the FLP recombinase region located within the SAT1-flipper cassette.

Primer	Sequence (5´-3´)
CpNDT80gen_F	GCCTTTTACATCTATCGAAGTCAAACTTG
Cp*UPC2*gen_F	CGCTATTCGTTTATTGTGTAGTCAAGC
FLP_R	AAG GAA AAA AGC GGC CGC TTT ATG ATG GAA TGA ATG GGA TG

In order to excise the SAT1 flipper cassete, transformants were grown in YPM (*Yeast Peptone Maltose*) medium (yeast extract 1%, peptone 2%, maltose 2%), at 30ºC, 180 rpm, overnight. From this, approximately $1x10^2$ recycled cells were plated onto YPD agar medium supplemented with Nou 20 µg ml^{-1}. Smaller colonies were chosen to screen for sucessfull cassette recycling.

Genomic DNA extraction

Genomic DNA extraction of *C. parapsilosis* transformants was carried out as described by Hoffman and Winston[17] , with certain modifications.

Approximately 1,5 ml of yeast culture grown in YPD medium at 30ºC overnight with agitation (180rpm) were transferred to a microtube and centrifuged at 20,000 *g* for 5 minutes. The supernatant was discarded and pellet was suspended in 200µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and briefly vortexed. Yeast suspension was then frozen at -80ºC for 5 minutes and immediately transferred to a 95ºC bath for 1 minute. This process was repeated at least two times and vortexed for 30 seconds. After adding 200 µl of chloroform, the mixture was then vortexed for 2 minutes with glass beads and centrifuged at 20,000 *g* for 3 minutes. The aqueous phase was then collected to a new microtube containing 400 µl of ice-cold absolute ethanol, mixed by inversion and incubated for 1 hour at -80ºC. The microtube was then centrifuged at 20,000 *g* for 5 minutes, and the pellet washed with 0,5 ml of 70% ethanol. Following another centrifugation, DNA was dried at 37ºC in order to remove any ethanol trace. Finally, DNA was resuspended in 50 µl in TE (10 mM Tris-HCl (pH 8), 1 mM EDTA) with 1 µl of RNAse 10 mg μl^{-1} and incubated for 1 hour at 37ºC. The quality and concentration was measured by espectophotometry (Nanodrop) and run in 0,8% agarose gel electrophoresis.

Confirmation of excision of the SAT1-Flipper cassette

Cassete excision was confirmed by PCR using the following pair of primers NDT80gen_up_F/NDT80_down_R and *UPC2*gen_up_F /*UPC2*down_R, respectively, for *NDT80* and *UPC2* genes. Genomic DNA from the clones grown in YPM was used as template. PCR reactions were prepared as described in **table 6**. Temperature profile of the PCR programmme comprised a denaturation step at 95ºC for 2 minutes, followed by 30 cycles of 95ºC for 30 seconds, 66ºC for 30 seconds and 72ºC for 3 minutes and a final extension step of 72ºC for 10 minutes, to screen for *NDT80* gene. For *UPC2* gene, PCR program used was similar with a minor difference, T_m was adjusted to 65ºC. PCR reactions were carried out in a BioRad MyCycler Personal Thermal Cycler®. PCR products were visualized after electrophoresis in 0,8% agarose gel.

Table 6. PCR reaction mix prepared for excision confirmation of the SAT1-Flipper cassette

Reagents	Stock Volume	Final Concentration
H_2O milliQ	Adjusted to 25 µl	
Buffer (10x)	2,5 µl	1x
dNTPs (10 mM)	0,5 µl	0.2 mM
Primer gen_F (100 pmol μl^{-1})	1 µl	4 pmol
Primer R (100 pmol μl^{-1})	1µl	4 pmol
DNA	1µl	100 ng μl^{-1}
Taq DNA polymerase (5U μl^{-1})	0,2µl	1U

SADH gene amplification/restriction

In order to confirm that clones obtained belong to the *C. parapsilosis sensu stricto*, amplification of the *SADH* gene was carried out, as described in Tavanti *et al*.[26]. Genomic DNA, used as template, together with the primers SADH_F and SADH_R (**Table 7**) and other components listed in **Table 8** were part of the PCR reaction. Temperature profile comprised a denaturation step at 95ºC for 2 minutes, followed by 30 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 30 seconds. The final extension step occurred at 72ºC for 10 minutes. PCR amplification reaction was carried out in a BioRad MyCycler Personal Thermal Cycler®. PCR product (716 bp) was visualized by electrophoresis in a 0,8% agarose gel. Then, was digested with 10 U of FastDigest® *BanI* enzyme (Fermentas®) in a 30 µl reaction for 5 minutes. Digested products were then run on a 0,8% agarose gel.

Table 7: Primers used for *C. parapsilosis SADH* gene PCR amplification.

Primer	Sequence (5´-3´)
SADH_F	GTTGATGCTGTTGGATTGT
SADH_R	CAATGCCAAATCTCCCAA

.

Table 8. PCR reaction mix prepared for *C. parapsilosis SADH* gene amplification

Reagents	Stock Volume	Final Concentration
H_2O milliQ	Adjusted to 25 µl	
Buffer (10x)	2,5 µl	1x
dNTPs (10 mM)	0,5 µl	0.2 mM
Primer F (100 pmol μl^{-1})	1 µl	4 pmol
Primer R (100 pmol μl^{-1})	1µl	4 pmol
DNA	1µl	100 ng μl^{-1}
Taq DNA polymerase (5U μl^{-1})	0,2µl	1U

.

Biofilm protocol assay

Biofilms were formed on commercially available presterilized, polystyrene, flat-bottom 12-well flat bottom plates. At first, 10 ml of cell suspension grown in Sabouraud Dextrose Broth medium was centrifuged at 5000 *g* and washed twice with sterile H_2O. Pellet was diluted in RPMI-1640 (Sigma-Aldrich®) medium to a concentration of approximately 1 x 10^6 cells/ml. From yeast suspension prepared, 1 ml was put into each well and incubated for 24 h, 48 h and 72 h at 35ºC. RPMI-1640 medium was used as a negative control.

Two different *in vitro* parameters were assessed to quantify biofilm formation, Crystal Violet (CV) and XTT (2,3-Bis-(2-Methoxyl-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) assays.

The protocol used for CV staining was adapted from O´Toole *et al.*[71]. In the first biofilm biomass quantification is carried out by adding 1 ml of 99% methanol to each well followed by an incubation for 15 minutes. Methanol is then removed and air-dried for 30 minutes. To stain the formed biofilm 0,02% of CV was added for 20 minutes, being after removed and washed twice with sterile H_2O. Water traces were then removed and biofilm was suspended in 1,5 ml of 33% acetic acid before quantification by spectophotometry at A_{590nm} in a UV-160 Shimadzu UV-Visible Recording Spectophotometer®.

The protocol described for the XTT assay was performed as described by Ramage *et al.*[70]. The colorimetric reduction of XTT by cellular enzymes measures biofilm metabolic activity. Formed biofilm was washed twice with PBS (Phosphate-Buffered Saline), followed by the addition of 1ml of XTT solution (25µl XTT (500mg/ml); 0,2µl Menadione 10 mM; 1ml PBS) to each well. Plates were then incubated for 3 hours in the

dark at 35ºC before spectophometric reading at A_{492nm} in a UV-160 Shimadzu UV-Visible Recording Spectophotometer®.

Yeast Flow Cytometric Microsphere Adhesion Assay

The microsphere adhesion protocol assay was performed as described by Silva-Dias *et al.*[39]. After growing *C. parapsilosis* BC014 and *NDT80/ndt80* strains on Sabouraud broth medium at 180 rpm at 30ºC overnight, 1ml of cell suspension was centrifuged for 4 min at 6000 *g* and ressuspended in 1ml of PBS.

Yeast suspension concentration was adjusted to approximately $2x10^6$ cells/ml in PBS; 200 µl of this suspension was mixed with 200 µl of microspheres (aprox. $2x10^7$ spheres/ml) in a test tube and incubated for 30 min at 35ºC with agitation. Samples were read in a flow cytometer on the fluorescence channel FL3. Cytometric assays were performed in a FACSCalibur cytometer (BD Biosciences, Sydney, Australia) standard model equipped with 3 photomultiplayers (PMTs), standard filters, and a 15-mW 488-nm Argon laser and using CellQuest Pro software (version 4.0.2). Results correspond to triplicate experiments.

Statistical analysis

Results were analyzed using Student's t test. *p* values <0.05 were considered statistical significant.

IX.Results/ Discussion

Disruption of *C. parapsilosis NDT80* and *UPC2* genes

In order to knockout *NDT80* and *UPC2* genes in the *C. parapsilosis* BC014 clinical strain it was used the SAT1-flipper cassette system. This technique uses a non-auxotrophic dominant resistance marker, *SAT1*, which confers resistance to nourseotricin, a member of the streptothricin group of antibiotics produced by *Streptomyces noursei*[49]. *SAT1* gene from the bacterial transposon Tn1825 encodes for a streptothricin acetyltransferase, which inactivates the antibiotic[48]. This cassete was first developed for *C. albicans* as described on Reuss *et al.*[22]. Some years later it was adapted to *C. parapsilosis* by Ding *et al.*[14]. SAT1-flipper cassete are constituted by the resistance gene referred above,

controlled by *ACT1* gene promoter; *FLP* gene, encoding for a recombinase, regulated by *MAL2* gene promoter, and flanked by two FRT (Flippase Recognition Target) (**Fig. 1**)

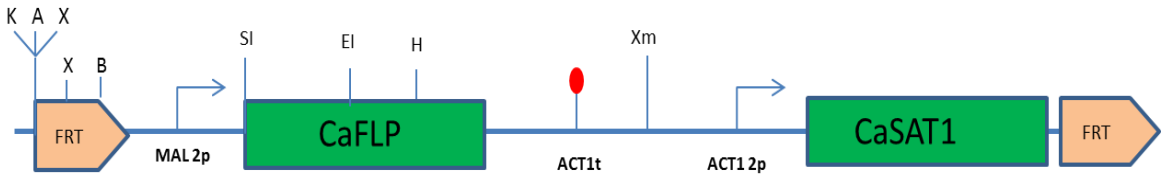

Figure 1. SAT1-flipper cassete (pCD8) scheme comprising *SAT1* gene (*CaSAT1*), nouseothricin resistance marker, and *FLP* gene (represented in the green boxes). Expression of these genes are controlled by *C. parapsilosis ACT1* (ACT1p) and *MAL2* (MAL2p) promoter regions (represented by the bent arrows), respectively. Transcription termination sequence of the *C. albicans ACT1* gene (ACT1t) is downstream *CaFLP* gene (filled circle).

After *C. parapsilosis* transformation, the resistance marker (*CaSAT1*) allows the selection of transformants that integrated the cassette into the genome, which enables their growth in the presence of nourseothricin. Since *C. parapsilosis* is a diploid yeast and in order to use the same selective marker to disrupt the second copy of the gene, *FLP* recombinase gene expression must be activated by growing transformants in maltose as a sole source of carbon. *FLP* activation induces cassette excision.

Gene disruption occurs by homologous recombination, therefore disruption cassette is constructed by cloning target gene flanking sequences in the unique restriction sites on both sides of the SAT1 flipper cassette. These sites are located both upstream and downstream of the cassete. In this study, we amplified the *C. parapsilosis NDT80* and the *UPC2* upstream and downstream sequences which were cloned on both ends of the SAT1 flipper cassette.

Cloning of these sequences into pCD8 plasmid were confirmed by PCR after the transformation in *E. coli* DH5α. PCR positive clones were sequenced.

C. parapsilosis transformation

Since *C. parapsilosis* is a diploid organism, the construction of homozygous mutants requires recycling of the selective marker after disruption of the first allele of the target gene to allow its subsequent use in a second round of transformation for inactivation of the remaining wild-type allele[21] (**Fig. 2**)

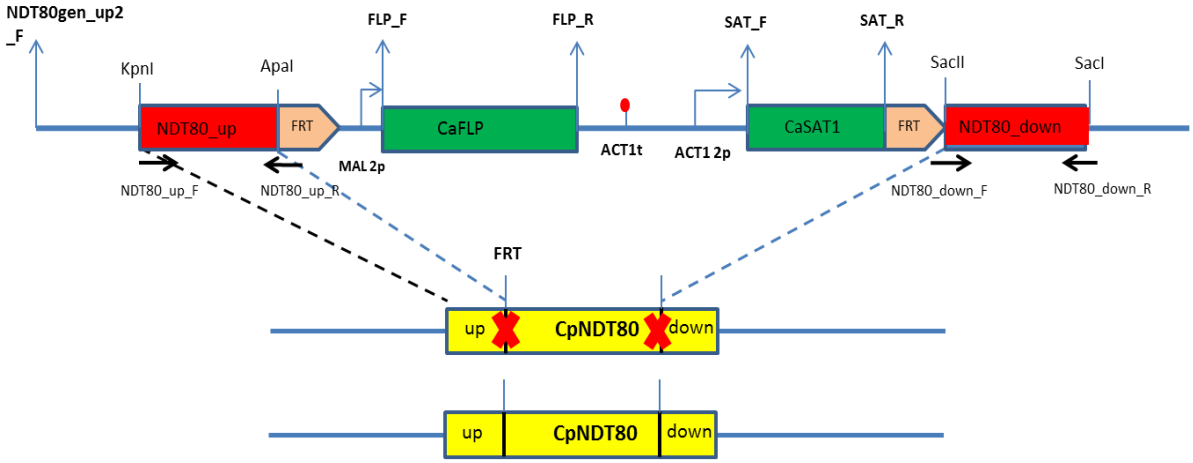

Figure 2. *C. parapsilosis NDT80* gene disruption by homologous recombination of the upstream and downstream sequences of the gene with its genomic target locus. Upstream and downstream regions were previously cloned in the SAT flipper cassette in th*pnI/ApaI* and *SacII/SacI* restriction sites respectively

The disruption cassetes pNG3 and pNG4 (containing upstream and downstream sequences of the *UPC2* and *NDT80* genes, respectively) were amplified by PCR and then transformed into *C. parapsilosis* BC014 by electroporation. From the transformation with pNG4 cassete resulted 47 Nourseotricin-resistant colonies after incubation for 2-3 days at 30ºC. These colonies were screened by PCR as described above in order to confirm pNG4 integration at its correct loci, and subsequently the deletion of one copy of the gene. The amplification of a 2.8 kb fragment in the clone Cp23 confirmed the integration of pNG4 at its target location (**Fig. 3**).

Transformation of BC014 with pNG3 cassette, used to disrupt the *UPC2* gene, originated 54 nourseotricin-resistant colonies. However, none of the clones obtained have integrated the cassette at *UPC2* gene locus.

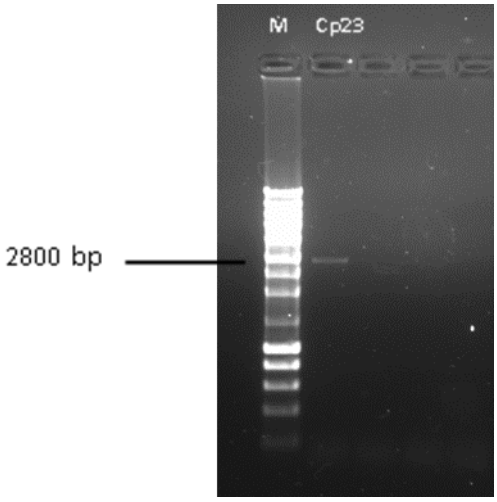

Figure 3 - Amplification of a 2.8 kb fragment confirming the integration of the pNG4 cassete at its target gene *NDT80*, and therefore its one copy delection, originating Cp23 clone. M represents the 100 bp Nzytech® ladder III.

pNG4 recycling

The excision of the cassette on this clone was done by growing Cp23 strain in YPM medium. This growth condition will induce the activation of *MAL2* promoter and consequently *FLP* recombinase activity. *FLP* recombinase will cleavage pNG4 cassete in FRT regions excising it from the genome. The excised clones were selected through their susceptibility to Nou 20 µg ml^{-1}. Nourseotricin susceptible colonies are the ones expected to have removed sucessfully pNG4 (**Fig. 4**).

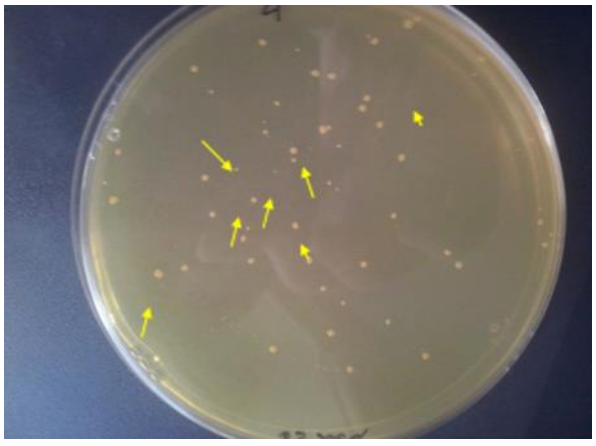

Figure 4. Selection of recycled pNG4 clones in Nou 20 µg ml^{-1}. Susceptible colonies to nouseothricin are chosen by its reduced size indicating growth inhibition.

After cassette recycling, six of the small transformants were plated onto YPD medium supplemented with Nou 200 µg ml^{-1} in order to reconfirm cassette excision. Three of the selected clones presented growth absence (Cp1, Cp2, Cp3) (**Fig. 5**)

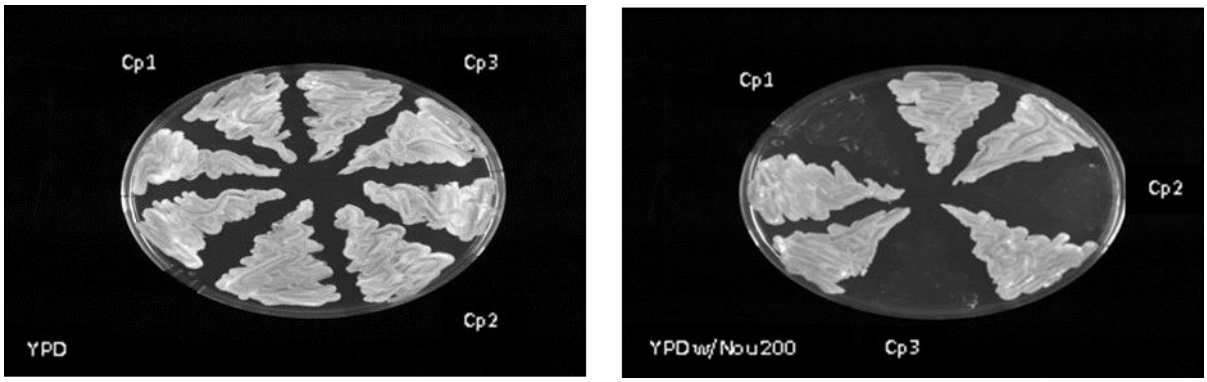

Figure 5. Confirmation of the pNG4 excision from *C. parapsilosis* BC014 genome. Clones were grown in YPD agar plate (A) and YPD agar plate supplemented with Nourseothricin 200µg ml^{-1} (B). Cp1, Cp2, Cp3 clones were susceptible to nourseothricin.

Furthermore, pNG4 recycling in these clones was confirmed by PCR using primers located in the genome flanking region of the *NDT80* gene and within the gene. As expected, two fragments were amplified in the PCR reaction. The band with approximately 3.0 kb represents the second allele with the intact gene, whilst the band with 1.1 kb correspond to the disrupted allele on the first transformation, containing only the upstream and downstream sequences of the *NDT80* gene (**Fig. 6**).

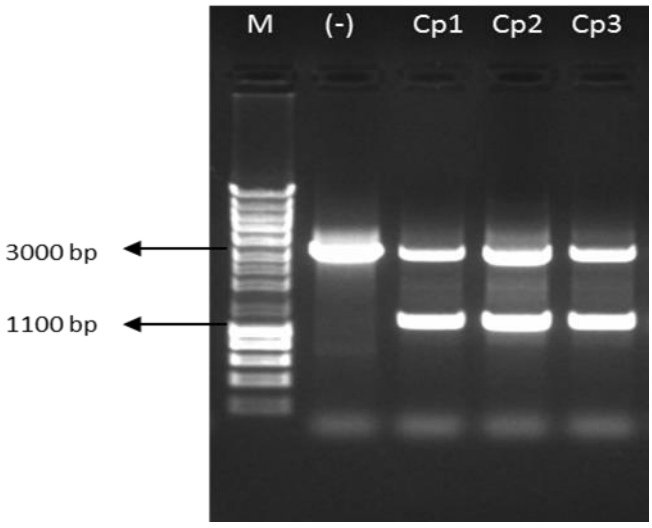

Figure 6. Confirmation of the disruption of one copy of the *NDT80* gene (*NDT80/ndt80*). An intact copy of the gene was amplified (3.0 kb fragment) and a fragment of approximately 1.1 kb, which corresponds to the upstream and downstream regions of the disrupted copy. A positive control was made with a BC014 strain. M represents the 100 bp Nzytech® ladder III.

Several transformations of the Cp23 clone (*NDT80/ndt80*) with the pNG4 cassete were carried out in order to obtain the homozygous deleted mutant. However, in thirty colonies obtained after transformation none of them was found to have pNG4 cassette in the *NDT80* locus, after PCR assessment. The difficulty faced in the deletion of the second copy of the *NDT80* gene as well as limitations of SAT1-flipper method is well documented on other studies[14,60].

The *NDT80* gene deletion would be successful if pNG4 cassette recombined to the upstream and downstream sequences of the target gene in the second allele, which did not happened on our experimental work. The most probable explanation for this result is sistematic recombination of the cassette on the upstream and downstream sequences present in the disrupted allele, leaving the second allele intact. This possibility was already reported when using SAT1 flipper cassette to disrupt other genes[60].

One possible solution to this limitation of the SAT1-flipper method would be to construct other cassette possessing upstream and downstream sequences more distant from the target gene than the ones contained in pNG4. This approach would avoid the recombination with upstream and downstream sequences in the disrupted allele.

Aneuploidy of *NDT80* gene increased yeast adhesion

Interestingly, *C. parapsilosis* Cp23 (*NDT80/ndt80*) grown in liquid media displayed a notorious increased adherence to abiotic surfaces, like glass, compared to the wild-type strain (BC014). Furthermore, Cp23 displayed a strong flocculation phenotype, indicating cell to cell adhesion (**Fig. 7**).

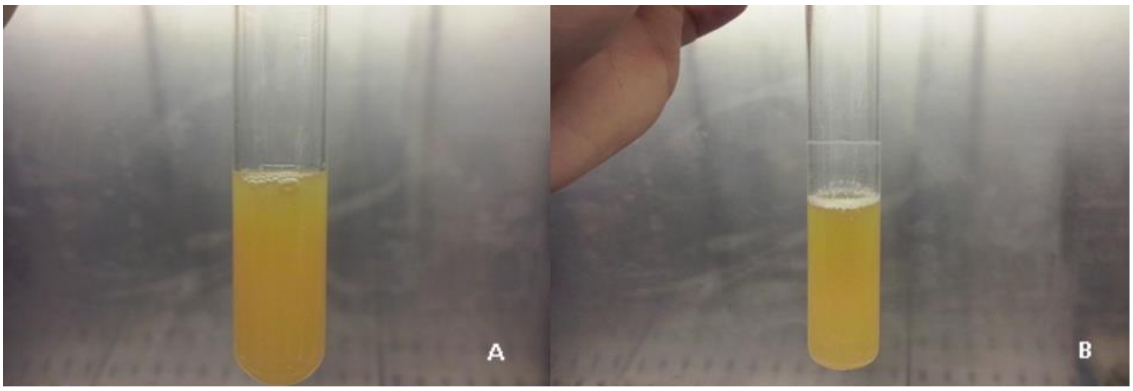

Figure 7. *C. parapsilosis* Cp23 (*NDT80/ndt80*) displayed increased cell to cell adhesion flocculating when growing in YPD liquid media (B) comparatively to the wild-type strain BC014 (A).

In the scope of the results obtained, yeast adhesion ability to polystyrene microspheres was quantified by flow cytometry. Statistical differences between *NDT80/ndt80* strain and wild-type were found in yeast adhesion profile. Interestingly, *NDT80/ndt80* strain displayed 5 fold increased adhesion to polystyrene than wild-type (**Fig. 8)**.

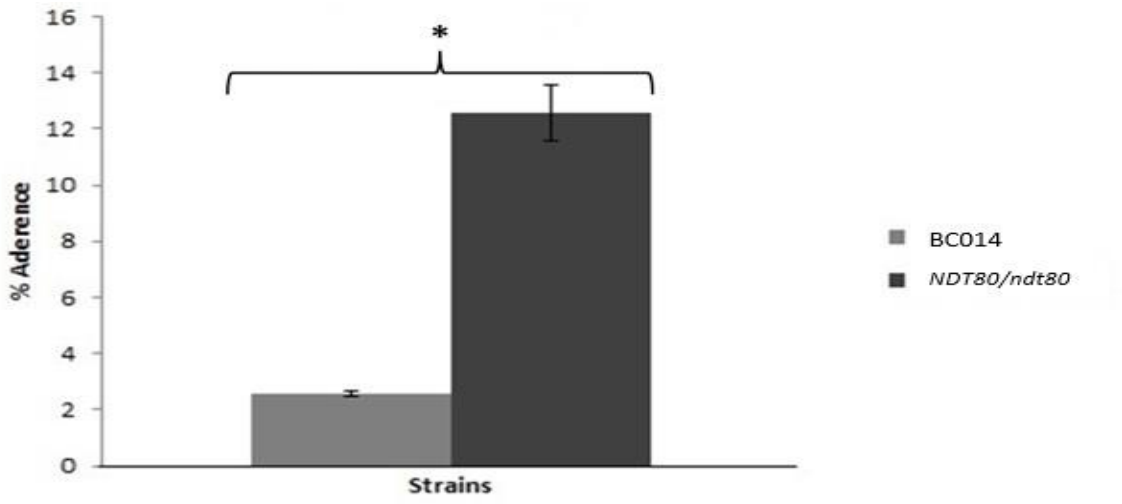

Figure 8. *NDT80* aneuploidy increases *C. parapsilosis* adhesion ability. Yeast adhesion ability to polystyrene beads was quantified using Flow Cytometric Microsphere Adhesion Assay. The *NDT80/ndt80* strain displayed approximately five times more adhesion than the wild-type (BC014) strain.

NDT80 aneuploidy triggers cell morphological changes

Deletion of one copy of the *NDT80* gene caused morphological cell phenotype shift. *C. parapsilosis NDT80/ndt80* cells are bigger and elongated in comparison with the mainly yeast form displayed by BC014 wild-type cells (**Fig.12**)

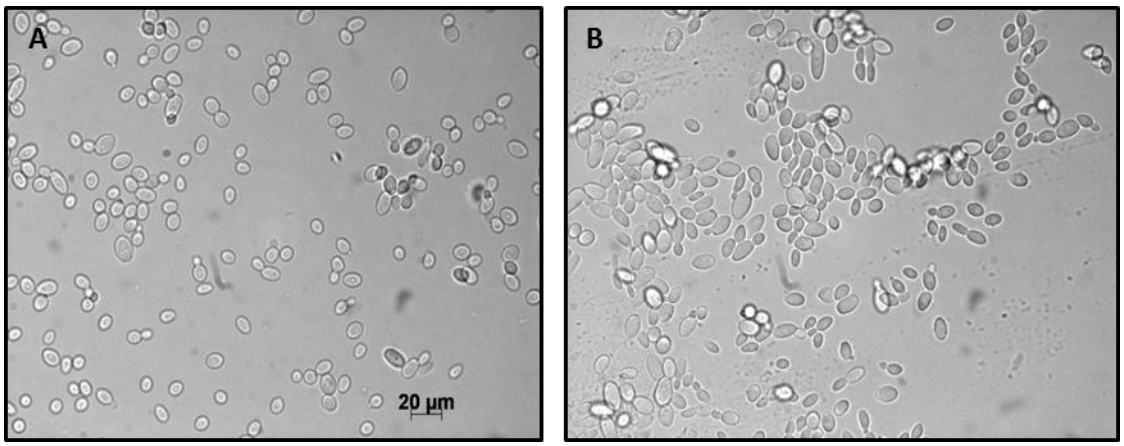

Figure 12 – *C. parapsilosis* morphological changes triggered by the deletion of one copy of *NDT80* gene. (A) BC014 strain – cells display a predominant round and small morphologic type; (B) *NDT80/ndt80* strain – cells present a bigger and elongated form. Cells were photographed after one day of growth on YPD agar plate at 35ºC.

In fact, *NDT80* gene has been described in *C. albicans* as an essential gene for the completion of the cell separation process, as a direct transcriptional regulator of genes encoding among others, the chitinase *Cht3p* and the cell wall glucosidase *Sun41p*[31]. In *C. albicans*, it was shown that the delection of *NDT80* gene caused cell separation defect.
Our results suggest that *NDT80* in *C. parapsilosis* may have a similar function as the one described for *C. albicans*. In fact, *NDT80/ndt80* cells are elongated and form agglomerates which could indicate a cell separation defect. Other recent studies in budding yeast show that the deletion of one copy of *NDT80* gene delays the meiotic commitment point. When compared to *NDT80/NDT80*, fewer *NDT80/ndt80* cells commit to meiosis in prometaphase I , suggesting that the commitment point shifted to the end of prometaphase I/beginning of metaphase I, describing that this gene can be involved in the whole meiotic committment process[62]**.**

SADH gene amplification

To exclude any possibility of contamination during experimental work, the amplification of *SADH* gene was carried out. Exclusive to *C. parapsilosis* species, *SADH* restriction profile provide a simple, rapid and accurate method of identification of the species belonging to this complex[52]. The 716 bp fragment of the gene *SADH* was amplified, confirming that it was a *C. parapsilosis* complex strain (**Fig.13-A**). The following restriction using the *BanI* endonuclease provides the identification within the complex, being the *C. parapsilosis sensu stricto* the far most common of the three subspecies.
After PCR product restriction with the *BanI* enzyme, it was clear that the strain with the deleted allele was a *C. parapsilosis sensu stricto* (*NDT80/ndt80*), once it displayed two bands, one of them corresponding to the restriction site of *BanI* (196 bp), as described for this species[66] (**Fig.13-B**).

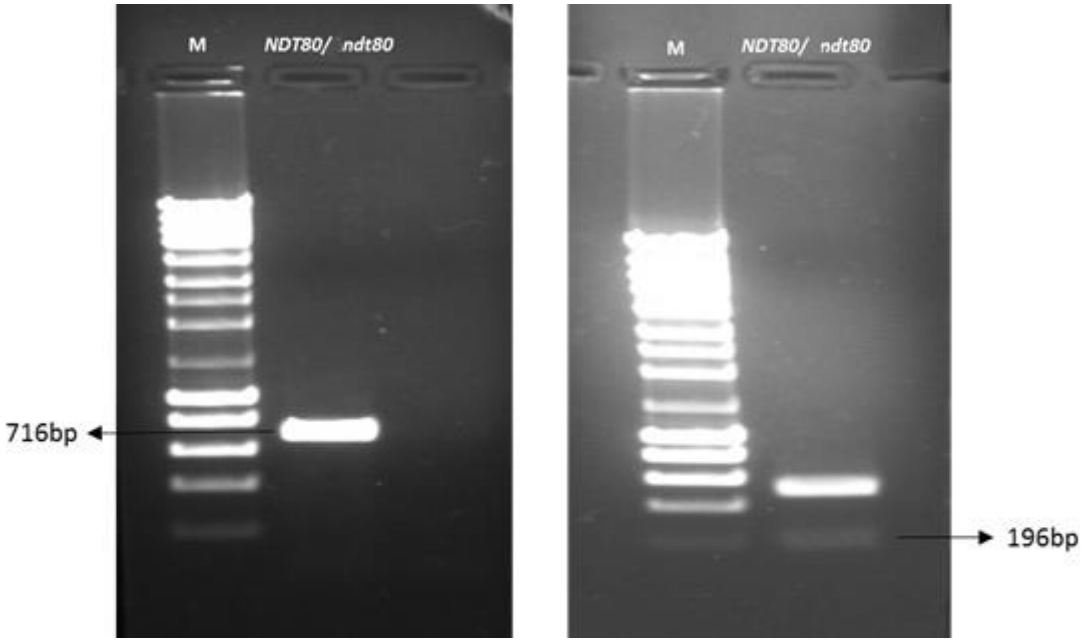

Figure 13. Cp23 (*NDT80/ndt80*) strain belongs to *C. parapsilosis sensu stricto* (A) PCR amplification of the 716 bp fragment of the *SADH* gene using as template Cp23 genomic DNA. (B) Restriction of the *SADH* gene with *BanI* resctriction enzyme: the band with approximately 196 bp represents the restriction site of *BanI* identifying *C. parapsilosis sensu stricto*. M represents the 100 bp Nzytech® ladder III.

Aneuploidy of *NDT80* gene increased biofilm formation

Microbial adhesion is considered the first step given by an organism to form a microbial community embebbed in an extracellular matrix named as biofilm. Thus, microorganisms that are able to adhere to abiotic surfaces as well as to each other, are particularly prone to form biofilm. Among other characteristics, biofilms constitute a reservoir for antifungal resistance development since it functions as a protection against antifungal drugs. Since Cp23 displayed increased adhesion, cell-cell and to abiotic surfaces, we assessed its ability to form biofilm.

Biofilm quantification was undertaken using two methodologies: Crystal Violet (CV) method, to assess the biomass, and the XTT reduction assay, to assess the metabolic activity.The CV method relies on the principle of staining the negative charged bacteria or polysaccharides[67], but not distinguish living and dead cells, whereas the XTT method uses a redox indicator to enumerate spectrophotometrically viable cells in biofilm. So, this methods complement each other in a certain way to evaluate the biofilm formation potential.

Only after one day of growth, it was visible to the naked eye differences between the Cp23 and wild-type strains. Only with 24 hours of incubation, *NDT80/ndt80* strain has formed a substantial amount of biofilm compared to the wild-type strain (**Fig. 9**)

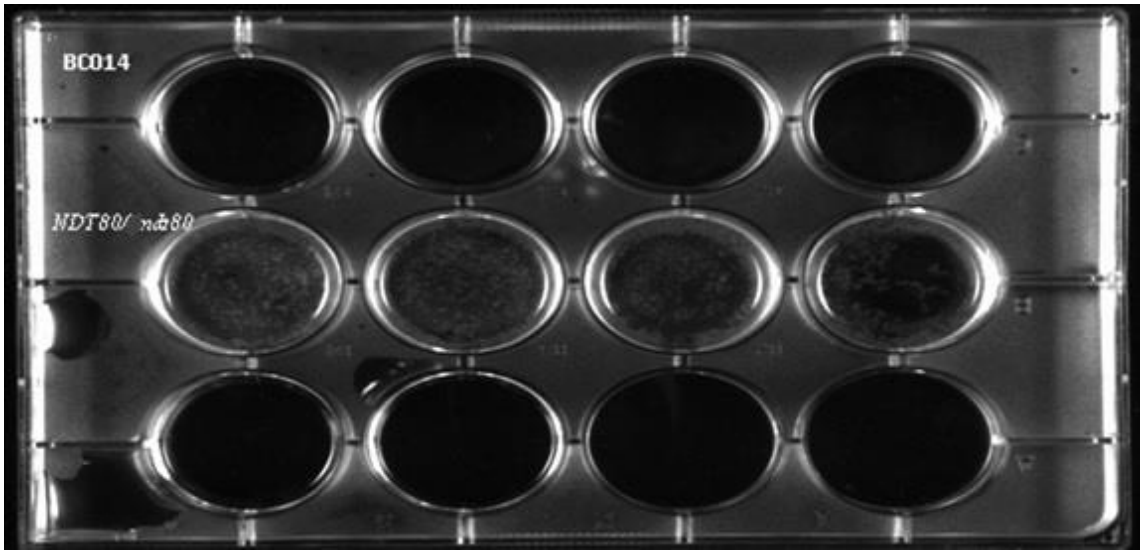

Figure 9 – Differences in biofilm formation between *NDT80/ndt80* and the wild-type strain after 24h of incubation at 35ºC on RPMI-1640 medium in a 12-well microplate.

For all the time frames (24 h, 48 h, 72 h), CV results shown that biofilms formed by Cp23 contained significantly (2 fold) more biomass than BC014 (**Fig. 10**). However, no differences were found when assessing metabolic activity, using the XTT method, between biofilms formed by the wild-type and Cp23, at 24 h and 48 h (**Fig. 11**).

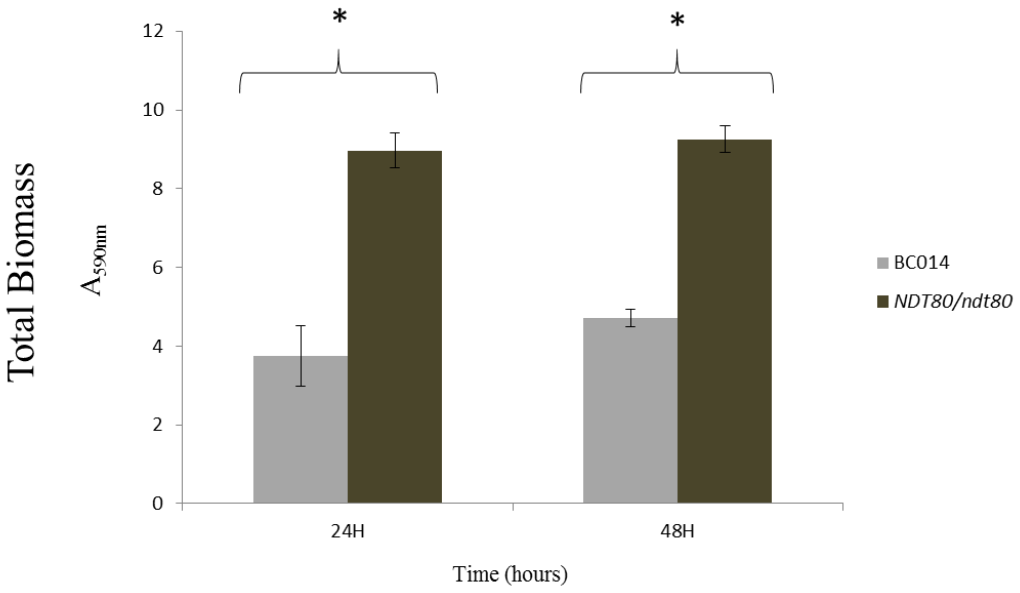

Fig. 10. Cp23 strain formed biofilms with increased amount of biomass than BC014. Biofilm formation was measuread using the CV method. Differences in biofilm formation found between Cp23 and wild-type were statistical significant at 24 h, 48 h and 72 h.

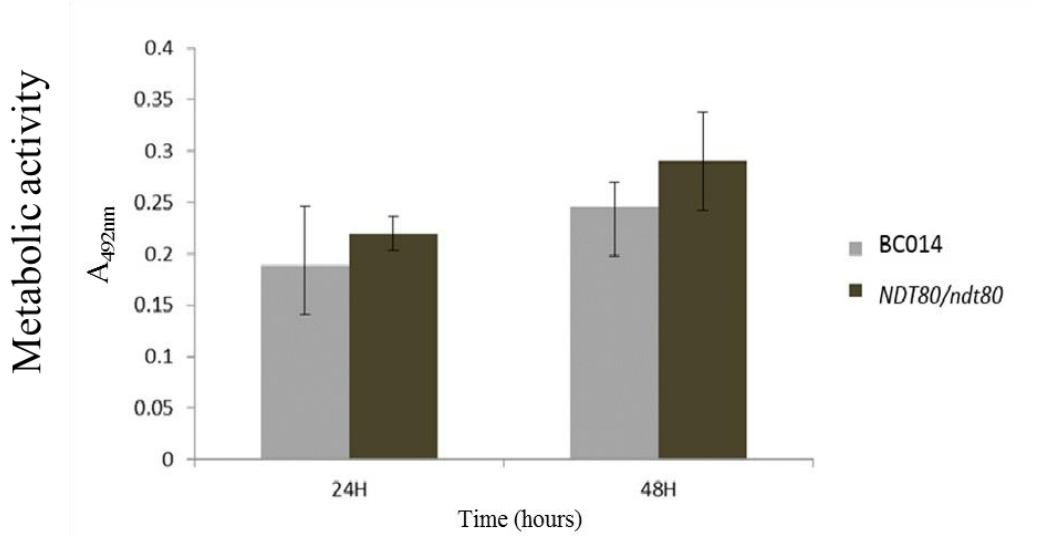

Fig. 11- Biofilm metabolic activity displayed by Cp23(*NDT80/ndt80)* and BC014. Biofilm metabolic was measuread using the XTT method. There were no significant differences found between *NDT80/ndt80* and the wild-type BC014 strain at 24 h and 48 h.

Interestingly and against what we have found in *C. parapsilosis*, delection of *NDT80* gene in *C. albicans* reveled to be detrimental for biofilm formation[63]. Moreover, it was shown that *NDT80* function on *S. cerevisae* and *C. albicans* is clearly distinguishable on both species, in the former, it regulates meiosis[64] and in the later, biofilm formation[65].

NDT80 and its role on *C. parapsilosis*

The efficiency of microbial adhesion has an huge impact at various levels, namely ecological, industrial, and clinical, with either positive or negative repercussions[40]. Pathogenic bacteria and fungal cells can adhere to host tissues or to biomaterial used in common medical indwelling devices leading to biofilm formation, which frequently results in bloodstream infections associated to high mortality rates[40,42].

It is known that *CaNdt80p* is required for the modulation of different biological processes, such as cell separation, hyphal growth, virulence, and azole sensitivity.

The findings on this experimental work are somewhat adjusted to the studies known to date on *C.albicans*. The delection of one allele of the TF *CpNDT80* is proven to have an role on important cell functions, namely on cell wall functions. The deletion of one

NDT80 allele has affected the biofilm formation and adherence potential of *C. parapsilosis*, being significantly different from the wild-type strain.

Results obtained indicate that *NDT80* gene is prone to be involved in adhesion, cell separation, hyphal growth, and virulence mechanisms on *C. parapsilosis*, as it was described on *C.albicans*[33].

The literature describes that *C. parapsilosis* and *C. albicans* may have the same capacity of adherence although with some variations[43], which suggest the same adherence mechanisms on both species. According to the studies done on *C.albicans*, *Ndt80p* is thought to act as a critical downstream effector promoting hypha formation and cell separation in response to different signals conveyed by different upstream pathways[31], being therefore described as a multifunctional general regulator[48]. During the iniciation of the hyphal formation process, increased levels of cAMP activate *Tpk2*, one of the protein kinase isoforms, will phosphorylate *Efg1*, an exclusive nucleus protein that interveins on the hyphal growth. *Ndt80* regulates the expression of this protein[68,69]. Moreover, in another study, gene expression microarray data comparing wild-type biofilms to deletion mutants of each regulator (one of them being *Ndt80*), it was proven that *Ndt80* on *C. albicans* bound about to approximately 273 of 999 genes (27%) of promoters involved in biofilm formation, and it is correlated with the other biofilm regulators such as *BCR1* and *BRG1*, forming an interconnected network of transcriptional regulators of biofilm development[68,69].

The deletion of the other *NDT80* allele will be necessary to further conclude the role of this TF on *C. parapsilosis*. After, a functional comparison could be made between *NDT80* role in *C. parapsilosis* and *C.albicans*.

Nevertheless, the knowledge of the genes involved on *C. parapsilosis* adhesion and biofilm formation is scarce.

X. Conclusion/Future perspectives

Throughout this experimental work, the main objective was partially achieved. It occurred a phenotypic change on the susceptible clinical strain BC014, when one allele of the *NDT80* gene was disrupted. This phenotypic change may compromise the whole morphology of the cell , which suggest an important role of this transcription factor not only on the azole-resistance mechanism but also on the biofilm formation capacity of *C. parapsilosis*. The deletion of the second allele of *NDT80* gene is of major importance in order to conclude definitely what is the role of the gene on the azole-resistance on this organism.

Once the knowledge of the molecular mechanisms underlying antifungal resistance in *C. parapsilosis* are scarce comparatively to what is already known for *C. albicans*, it is critical to understand the role of the TF such as *UPC2* and *NDT80* to further establish a parallel with other species of *Candida* spp.

XI. Bibliography

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